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Florid plaques in ovine PrP transgenic mice infected with an experimental ovine BSE

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The occurrence of the variant Creutzfeldt–Jakob disease (vCJD), related to bovine spongiform encephalopathy (BSE), raises the important question of the sources of human contamination. The possibility that sheep may have been fed with BSE-contaminated foodstuff raises the serious concern that BSE may now be present in sheep without being distinguishable from scrapie. Sensitive models are urgently needed given the dramatic consequences of such a possible contamination on animal and human health. We inoculated transgenic mice expressing the ovine *PrP* gene with a brain homogenate from sheep experimentally infected with BSE. We found numerous typical florid plaques in their brains. Such florid plaques are a feature of vCJD in humans and experimental BSE infection in macaques. Our observation represents the first description, after a primary infection, of this hallmark in a transgenic mouse model. Moreover, these mice appear to be a promising tool in the search for BSE in sheep.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) are neurodegenerative diseases characterized by vacuolation and accumulation of the pathological form of the prion protein—PrPsc—in the brain. Ten years after the first bovine spongiform encephalopathy (BSE) cases reported in the United Kingdom, the occurrence in April 1996 of the variant form of the human TSE Creutzfeldt–Jakob disease (vCJD), affecting unusually young patients, raised the important question of the sources of human contamination (Will *et al.*, 1996; Almond and Pattison, 1997). The histopathological hallmark in the brain of all patients with vCJD is the presence of numerous florid plaques (Fraser, 1979; Ironside and Bell, 1997) that were also found in the brain of macaques infected with cattle BSE (Lasmezas *et al.*, 1996), thus

providing the first link between BSE and vCJD. This correlation has been rapidly confirmed by disease transmission studies in inbred wild-type mice or bovine PrP transgenic mice (Will *et al.*, 1996; Almond and Pattison, 1997; Bruce *et al.*, 1997; Scott *et al.*, 1999). The similarities between incubation times and between the lesion distributions revealed that a common agent was responsible for both vCJD in human and BSE in cattle (Bruce *et al.*, 1997; Scott *et al.*, 1999). Moreover, studies of the quantitative ratios of the different PrPres glycoforms and evaluation of the molecular mass of the prion protein showed a typical and unusual molecular pattern named type 4 (Collinge *et al.*, 1996; Hill *et al.*, 1997) in all cases of vCJD compared with sporadic and iatrogenic CJD. This type 4 pattern has also been found in macaques experimentally infected with the BSE agent (Collinge *et al.*, 1996; Lasmezas *et al.*, 2001).

The origin of BSE remains unknown. It may have arisen from sheep TSE, scrapie, through the recycling of scrapie agent in meat and bone meal (MBM) used in cattle foodstuff. These two types of TSEs are, however, distinct in several respects. Natural sheep scrapie is rather common in sheep and has been present in sheep flocks for a long time whereas TSEs were unknown in cattle prior to the 1980s. While BSE seems to be sustained by a single major strain of agent (Bruce *et al.*, 1997), natural scrapie is highly polymorphic with several scrapie strains that differ in terms of incubation time and lesion profile following transmission in inbred wild-type mice (Fraser and Dickinson, 1968; Fraser and Dickinson, 1973).

Since BSE can be transmitted experimentally to small ruminants, including by oral routes (Foster *et al.*, 2001), there is a risk that BSE may have been disseminated through MBM foodstuff into the sheep population. Subsequent horizontal and vertical transmission of the BSE agent within sheep population may

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constitute a new source of BSE agent exposure to human. Sensitive models are urgently needed considering the dramatic consequences on animal and human health. Biochemical analysis may be discriminant (Hill *et al.*, 1998) but there is, at least, one exception with the CH1641 experimental scrapie showing the same electrophoretic pattern as that found in a BSE-infected sheep. In addition, the lesion profile, following transmission of the disease to wild-type mice, is a time-consuming method. Thus, there is unfortunately no real specific and easy manner to recognize rapidly the BSE agent in sheep.

To address this question we have used ovine PrP transgenic mice [Tg(OvPrP4)] expressing, selectively in their brain, the ovine PrP gene using the neuron-specific enolase promoter (Crozet *et al.*, 2001). Compared with wild-type mice, these mice are more sensitive to sheep scrapie (Crozet *et al.*, 2001). In the present study, we have inoculated Tg(OvPrP4) mice with a brain homogenate prepared from a sheep inoculated with French cattle BSE agent (Baron *et al.*, 2000). Here we report that the singular 'florid plaques' signature in the brain of infected Tg(OvPrP4) mice is identical to that of vCJD in humans. This simple criteria may thus provide the opportunity to identify possible field contamination of sheep by the BSE agent.

RESULTS AND DISCUSSION

We used Tg(OvPrP4) mice expressing the ovine PrP gene ($AA_{136}RR_{154}QQ_{171}$ genotype) in the brain under the control of the neuron-specific enolase promoter (Crozet *et al.*, 2001). Five-week-old mice ($n = 20$) were intracerebrally inoculated with a brain homogenate obtained from a sheep ($AA_{136}RR_{154}QQ_{171}$ genotype) experimentally infected with cattle BSE agent (Baron *et al.*, 2000). Two hundred and twenty days after inoculation, the mice started to display extensive clinical signs of encephalopathy (leanness, hunched posture, hindlimb paralysis, equilibrium trouble, plastic tail and prostration), and they died between 221 and 371 days post-inoculation (p.i.) (mean survival time 300 ± 40 days). Biochemical analysis, performed on every other mouse brain, showed the presence of proteinase K-resistant PrP with an electrophoretic profile comparable to that of ovine BSE (Figure 1). When comparing the electrophoretic features obtained in mice with the natural scrapie isolates, a lower apparent molecular weight of the unglycosylated PrPres was observed in Tg(OvPrP4) mice inoculated with ovine BSE. This difference is evocative of the one observed between PrPres in
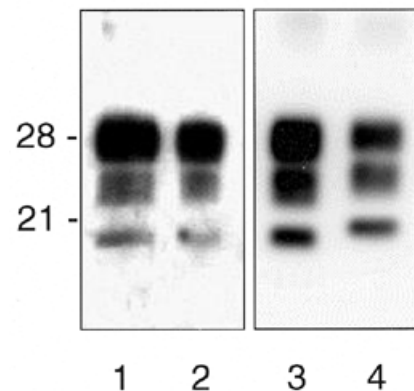

Fig. 1. Western blot analysis with RB1 antibody showing similar electrophoretic profiles of the proteinase K-resistant protein in the brain of BSE-infected sheep (lane 1) and in the brain of Tg(OvPrP4) mice (lane 2). This electrophoretic pattern shows a lower apparent molecular weight of proteinase K-resistant protein in the brain of Tg(OvPrP4) inoculated with ovine BSE (lane 3) compared with Tg(OvPrP4) inoculated with a natural scrapie isolate (lane 4).

sheep inoculated with BSE and other natural scrapie isolates (Hill *et al.*, 1998; Baron *et al.*, 2000) (Figure 1) and also of those described in mice (Kuczius *et al.*, 1998) or in humans with the so-called type 4 pattern in vCJD (Collinge *et al.*, 1996). This already suggests that the original biochemical BSE strain properties, characterized by a specific electrophoretic type 4-like pattern, are maintained in infected Tg(OvPrP4) mice, even after a passage in sheep. Beside these biochemical features, histological analysis on the other mouse brains showed spongiform changes (Figure 2A). PrPsc immunostaining consisted of fine, granular deposits, always associated with vacuolation in the habenula, thalamus and hypothalamus (Figure 2B). More predominantly, plaques with a fibrillar core were observed, isolated or sometimes grouped into small clusters (Figure 2C). Remarkably, these plaques were surrounded by a ring of small vacuoles reminiscent of florid plaques (Figure 2C). In addition, on adjacent sections, Congo Red staining (Figure 2D) demonstrated the amyloid nature of these plaques, further providing evidence that they are similar to the florid plaques described for vCJD in human (Ironside and Bell, 1997). These florid plaques were numerous (Table I) and were found mostly in the subcallosal

Table I. Breed and genotype of sheep used for Tg(OvPrP4) mice inoculation

TSE	Breed	Genotypes	Mean survival time (days ± SEM)	Number of florid plaques per slide
Experimental BSE in sheep	Lacaune	ARQ/ARQ	300 ± 40	120 ± 30
Natural scrapie	Lacaune	ARQ/ARQ	303 ± 74	0
Natural scrapie	Blanc du massif central	ARQ/ARQ	238 ± 7	0
Natural scrapie	Manech Tête Rousse	ARQ/VRQ	290 ± 104	0
Natural scrapie	Manech Tête Rousse	VRQ/VRQ	370 ± 124	0
Natural scrapie	X Texel	VRQ/VRQ	361 ± 93	0
Natural scrapie	Charollais X Texel	VRQ/VRQ	502 ± 133	0

Mean survival time and total number of florid plaques per brain section in Tg(OvPrP4) diseased mice of each experiment.
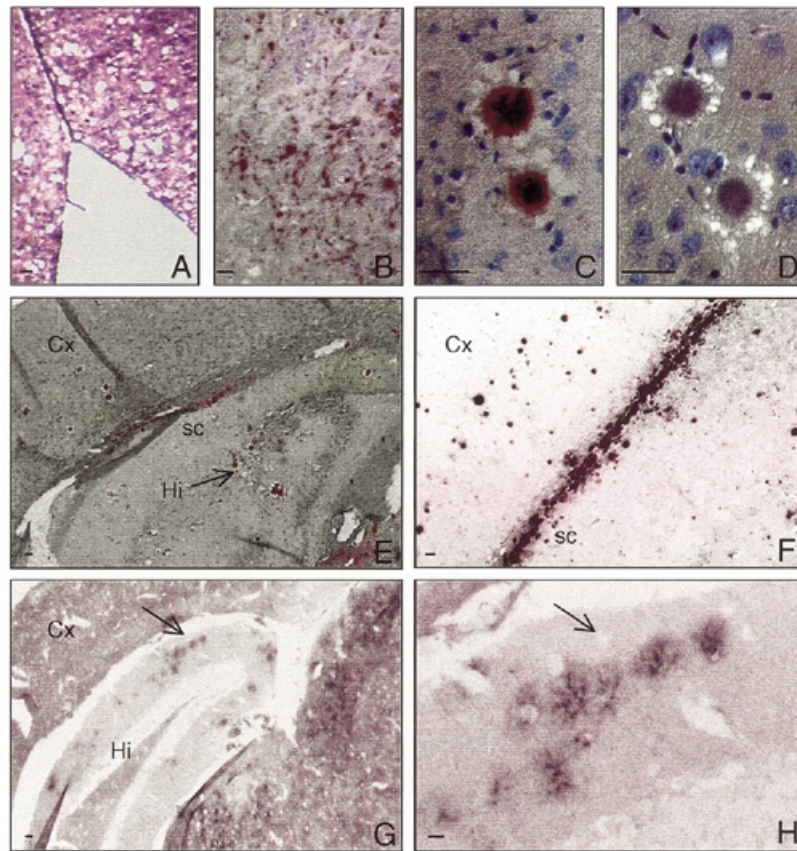
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Fig. 2. (**A**) Hematoxylin–eosin staining showing the presence of vacuolation lesion in the hypothalamus of Tg(OvPrP4) mice infected with 'ovine BSE'. (B, C and E–H) PrP immunostaining using SAF 84. It consists of parenchymatous and fine granular red deposits like in the thalamic nuclei (**B**) or in a plaque-type staining (**C**) showing a fibrillar core surrounded by a regular ring of vacuoles. The amyloid nature of these plaques is confirmed by Congo Red coloration (**D**). (**E** and **F**) These florid plaques are most frequently seen in the cortex (Cx), subcallosal region (sc) and hippocampus (Hi, arrow). (**G** and **H**) In the same area of wild-type mice inoculated with 'ovine BSE', PrPsc immunohistochemistry shows fine granular black deposits but not florid plaque-type accumulation. Scale bar, 20 μm

region, hippocampus, midbrain and thalamus, and moderately in the cortex and dorsal medulla (Figure 2E and F).

The anatomical distribution of PrPsc florid plaques observed here, as early as the first passage, is similar to the amyloid plaque distribution reported in second passage experiments for bovine PrP transgenic mice infected either with vCJD or BSE (Scott *et al.*, 1999). As a different transcription promoter was used in this last case (endogenous PrP promoter), these similarities suggest that the PrPsc plaque deposit topography depends more on the strain of agent than on the PrP gene expression pattern. Until now, florid plaques were never detected in Tg(OvPrP4) mice (*n* = 45) inoculated with six different isolates of scrapie (Table I), indicating that this hallmark is peculiar to BSE agent transmitted to ovine species.

The biological meaning of florid plaques remains unclear. Originally described in wild-type mice inoculated with three Icelandic sheep (Fraser, 1979, 1983) and in a mouse adapted-scrapie strain (111A) (McBride *et al.*, 1988), these florid plaques were rare in sporadic CJD (Kopp *et al.*, 1996). No florid plaques were found in other cases of wild-type mice inoculated from field scrapie cases. This shows that this phenomenon is probably limited to a very narrow range of scrapie strains and is not a usual feature. However, a high density of florid plaques has been

reported only for vCJD and has been a decisive criterion in establishing a similar origin in these patients (Will *et al.*, 1996; Ironside and Bell, 1997). This neuropathological marker of vCJD has also been described in macaques experimentally infected with BSE, producing a disease close to vCJD in humans and providing the first link between the BSE agent and the vCJD disease (Lasmezas *et al.*, 1996). In addition, in control experiments still in progress over 500 days p.i., consisting of the inoculation of a BSE isolate in Tg(OvPrP4) mice, only six mice have died so far (437 ± 104 days p.i.). First, biochemical and histochemical analysis did not allow the detection of PrPsc accumulation, suggesting that cattle BSE is not easily transmissible to Tg(OvPrP4) mice. This probably reflects the species barrier effect in this transmission. Florid plaques have never been observed after transmission of BSE or vCJD to mice, even when the mice carried a human or bovine *PrP* transgene (Collinge *et al.*, 1995; Bruce *et al.*, 1997; Hill *et al.*, 1997; Lasmezas *et al.*, 1997; Scott *et al.*, 1999). In previous studies, sheep experimentally infected with BSE inoculated to wild-type mice also failed to produce florid plaques. Similarly, in our control study, still in progress, wild-type mice inoculated with our ovine BSE isolate died from 348 days p.i. and >500 p.i. with spongiosis and PrPsc accumulation, but without florid plaques

(Figure 2G and H). Thus, another factor—present in our ovine transgenic mice and common at least to some humans or macaques—could be decisive for the manifestation of the BSE agent into florid plaques. In any case, the correlation between the abundance of florid plaques and BSE contamination remains to be clarified. Since we found a high density of florid plaques in Tg(OvPrP4) mice inoculated with the brain of sheep experimentally infected with BSE, and none in any of the mice inoculated with different scrapie isolates, the presence of florid plaques could be considered in our model as a criterion to discriminate BSE from natural scrapie in sheep.

The sheep experimentally infected with BSE was of the $AA_{136}RR_{154}QQ_{171}$ genotype. The fact that this sheep and Tg(OvPrP4) mice share the same PrP genotype could be important for the occurrence of florid plaques. Tg(OvPrP4) mice, however, were sensitive to natural scrapie isolated from $AA_{136}RR_{154}QQ_{171}$ as well as $VV_{136}RR_{154}QQ_{171}$ and $AV_{136}RR_{154}QQ_{171}$ genotypes (Table I), and the genotype did not seem to influence the nature of the PrPsc deposits. We did not attempt to investigate the effect of PrP genotype on florid plaques occurrence, for which brain homogenates of other BSE-infected sheep would have been required.

Taken together, these results already provide an important improvement to prion strain identification in sheep. Compared with wild-type mice, our Tg(OvPrP4) mice are more susceptible to sheep scrapie isolates (Crozet *et al.*, 2001) and seem to be able to discriminate BSE agent in sheep. Indeed, the easy recognition of florid plaques following relatively short incubation periods may be an advantageous alternative to the lesion profile method performed in wild-type mice. Finally, the proper calculation of sensitivity and specificity of florid plaques criterion would require testing of other sheep brain homogenates experimentally infected with BSE (Foster *et al.*, 2001), as well as a larger number of field and experimental scrapie isolates.

Speculation

The appearance of florid plaques in future inoculations of Tg(OvPrP4) mice with field scrapie could be interpreted in two ways. It might reveal BSE contamination in sheep. Alternatively, it might allow the identification of a natural scrapie strain similar to those that could have been at the origin of the BSE epidemic. In both cases this would be important, as it would allow the identification of a sheep population potentially hazardous for human health.

METHODS

Animals. The Tg(OvPrP4) transgenic mice used in this study selectively expressed in neurons the ovine PrP gene ($AA_{136}RR_{154}QQ_{171}$ genotype) but not the endogenous murine PrP. The generation of these mice and their susceptibility to scrapie are described in Crozet *et al.* (2001). The transgenic mice were cared for and housed in an autonomous filtered pressurized enclosure according to the guidelines of the French Ethical Committee (Decree 87-848) and European Community Directive 86/609/EEC.

Animal inoculation and follow up. The transgenic 5-week-old mice were inoculated intracerebrally with 20 μl of a 10% (wt/vol)

brain homogenate in 5‰ glucose, from Lacaune sheep with an AA_{136} RR_{154} QQ_{171} that had been experimentally infected with cattle BSE ($n = 20$) and with six natural scrapie isolates ($n = 10$ per experiment) from different regions in France detected by the French Epidemiological Surveillance Network (D. Calavas, AFSSA-Lyon, France) (Table I). The mice were then checked weekly for the presence of clinical signs such as leanness, hunched posture, hindlimb paralysis, equilibrium trouble, plastic tail, prostration, tremors, ruffled fur, abnormal gait and clasping feet.

As soon as a mouse showed one of these clinical signs, it was isolated and monitored daily, until death. Mice were killed when the intensity of clinical symptoms appeared life threatening or in some cases were found dead. The whole brain was quickly removed, and the brain of every second mouse was fixed [paraformaldehyde, 0.1 M phosphate-buffered saline (PBS) pH 7.4] for immunohistochemical analysis, the other frozen samples being stored at –80°C for western blot analysis as described elsewhere (Crozet *et al.*, 2001).

Detection of abnormal PrP in scrapie-inoculated transgenic mice. After being fixed in paraformaldehyde in 0.1 M PBS pH 7.4 for one night at 4°C, the brains were dehydrated through graded alcohols and embedded in paraffin. Sections (5 μm) were collected onto pre-treated glass slides (Polylysin or StarFrost, Fischer Scientific) and baked overnight at 57°C. The slides were then dewaxed and were submitted to pre-treatments designed to destroy the normal cellular PrP. These consisted of a formic acid treatment (98%; Merck) for 10 min at room temperature, 121°C for 30 min in water, each step being followed by a 5 min rinse in water, which appeared more appropriate for detection of PrPsc by immunohistochemistry. The slides were treated with proteinase K (20 μg/ml; Roche-Boehringer) for 10 min at 37°C. All following steps were performed at room temperature (22–24°C). Endogenous peroxidase activity was inhibited with 2% H_2O_2 (Merck) in 0.1 M PBS for 5 min. Non-specific antigenic sites were blocked by a 30-min incubation in blocking reagent (Roche-Boehringer). SAF84 monoclonal antibody recognizing the human 142–160 PrP sequence (0.5 μg/ml) (kindly provided by Dr J. Grassi, CEA/SPI, Saclay, France) was then applied overnight. The brain sections were rinsed before detection of the primary antibody using the ABC system (Vector). These steps were followed by rinsing in 0.1 M PBS, and the peroxidase was finally revealed by incubating the sections in 0.1 M PBS containing aminoethylcarbazole (AEC; Dako) or diaminobenzidine (DAB; Clinisciences) to give red or black deposits, respectively. The slides were weakly counterstained with aqueous hematoxylin before mounting (GelMount).

Congo Red staining. Congo Red staining was used to reveal specifically the amyloid nature of the plaques. Dewaxed slides were coloured with Harris hematoxylin for 10 min. Congo Red (1%, 30 min), lithium carbonate (15 s), 80% ethanol (30 s) were then applied successively. The slides were dehydrated and mounted.

Western blot analysis of PrPres. The presence of protease-resistant protein was analysed by western blot analysis using the RB1 monoclonal antibody raised against synthetic bovine 105–120 PrP peptide as described elsewhere (Madec *et al.*, 1997; Crozet *et al.*, 2001).

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